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Full Length Article

Loop-Mediated Isothermal Amplification Targeting *OMP P2* Gene for Detection of *Haemophilus parasuis*

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Abstract

This study aimed to develop a rapid, highly sensitive and specific detection method of *Haemophilus parasuis* (*Hps*) utilizing loop-mediated isothermal amplification (LAMP). According to highly conserved sequences and six distinct regions of *OMP P2* gene published in the GenBank, four specific primers were designed. The isothermal amplification of DNA templates were performed and catalyzed with *Bst* DNA polymerase. In addition, the LAMP reaction conditions (including primer ratio, concentrations of magnesium sulphate ($MgSO_4$), dNTP and *Bst* DNA polymerase, reaction time and temperature) were optimized, and the sensitivity of LAMP were investigated by comparing with that of PCR. The results revealed that the developed LAMP method for detection of *Hps* had high specificity and sensitivity. The LAMP reaction could be accomplished within 45 min and its minimum detection concentration for DNA was 0.2 pg/μL. Thus, the LAMP is a rapid, efficient and simple method for the detection of *Hps* and has good prospects for application in grass-roots sectors and the laboratory. © 2018 Friends Science Publishers

Keywords: *Haemophilus parasuis*; Symbiotic bacteria; *OMP P2* gene; Loop-mediated isothermal amplification

Introduction

The *Hps* is a kind of symbiotic bacteria and is commonly parasitic on the swine upper respiratory tract without clinical symptoms. It can be isolated from the nasal cavity, tonsil and tracheal anterior segment. However, *Hps* can invade the body and cause severe systemic diseases including fibrinous polyserositis, arthritis and meningitis under certain conditions (Oliveira *et al*., 2003; Olvera *et al*., 2006). Due to the mixed infection of *Hps* with other swine pathogens, the incidence of *Hps* disease is increasing around the world in recent years. It has made great economic losses in the swine industry (Oliveira *et al*., 2004). Therefore, the establishment of a rapid, efficient and simple detection method of *Hps* is important to prevent and cure the *Hps* disease successfully.

The LAMP technology developed by Notomi a novel DNA amplification method (Notomi *et al*., 2000). The reaction principle of LAMP is as follows: (1) The primers (F3, B3, FIP and BIP) are designed according to the conserved sequences and they can recognize the six distinct sequences of the target gene; (2) under isothermal conditions, the *Bst* DNA polymerase can cause the self-circulation chain replacement reaction of DNA; (3) after amplification for 60 min at 60–65℃, the target sequences can be amplified by more than 109 times. The LAMP products are stem-loop DNA at different sizes and show ladders on agarose gel electrophoresis. Due to recognizing the six distinct sequences of the target gene, the LAMP reaction has high specificity. Comparing with expensive thermal cycler for PCR, LAMP doesn't possess facility limitation because it just requires a stable heat equipment such as water bath.Thus, the LAMP is also low cost.

In this study, four specific primers were designed based on the conserved regions of *OMP P2* gene of *Hps* and the target genes were amplified. Also, the sensitivity and specificity of LAMP were investigated. A rapid, efficient and simple detection method of *Hps* has been developed and it has good prospects for application in grass-roots sectors and the laboratory.

Materials and Methods

Main Reagents and Strains

Bst DNA polymerase was purchased from the New England Bio-Technology Co., Ltd. (Beijing, China). Magnesium sulphate and dNTPs were obtained from the Beijing Dingguo Changsheng Bio-Technology Co. Ltd. (Beijing, China). DNA Marker DL2000 was acquired from the Beijing Nobleryder Bio-technology Co., Ltd. (Beijing, China).

Hps strains were purchased from the China Institute of Veterinary Drugs Control (Beijing, China). *Actinobacillus pleuropneumoniae*, swine pseudorabies virus, *Escherichia*

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coli, *Salmonella*, *Staphylococcus aureus*, *Streptococcus agalactiae*, porcine parvovirus, *Transmissible gastroenteritis virus* and *Mycoplasma hyopneumoniae* were supplied by the Infectious Diseases Laboratory of the College of Traditional Chinese Veterinary Medicine in the Agricultural University of Hebei.

Primers

Based on six distinct regions of *OMP P2* gene (NZ_ABKM01000007.1) published in the GenBank and the LAMP primer designing principle, four specific primers were designed using the Primer Explorer 3.0 software (http://www. netlaboratory.Com). Primers sequences for LAMP are shown in Table 1 and Fig. 1.

According to the sequences of *OMP P2* gene published in the GenBank, the PCR primers (P1: 5′-TGAACATCACCACCGAAT-3′; P2: 5′-TCTTAGATACACCAGGACACG-3′) were designed, and the target fragments that were expected to be amplified were 262 bp in size.

Extraction of Bacterial Genomic DNA

The total DNA of *Hps* was extracted according to instructions of DNA extraction kits obtained from the Beijing Sunbiotech Co., Ltd. (Beijing, China).

PCR Reaction

The volume of the PCR reaction system was 50.0 μL mixture containing 1.0 μL each primer,4.0 μL dNTP (25 mmol/L), 2.0 μL template DNA, 0.5 μL Taq DNA polymerase (2.5 U), 3.0 μL magnesium (25 mmol/L),5.0 μL 10×PCR buffer and ddH_2O, which was added to the final amount of 50.0 μL. The PCR temperature profiles were as follows: initial denaturation at 94.0℃ for 2 min; followed by 35 cycles of denaturation at 94.0℃ for 1 min, annealing at 52.0℃ for 1 min, and extension at 72.0℃ for 1 min; final extension at 72.0℃ for 5 min. In order to ensure the specificity of PCR products, a total of 10.0 μL PCR products were sequenced and analyzed by Beijing Sunbiotech Co. Ltd. (Beijing, China).

Optimization of LAMP Reaction System

The LAMP reaction conditions including the ratio of the inner primer to the outer primer and concentrations of $MgSO_4$, dNTP and *Bst* DNA polymerase were optimized. The LAMP reaction was performed in a 25.0 μL reaction system containing 2.0 μL DNA templates, 2.5 μL 10 × buffer, outer primer, inner primer, dNTPs, $MgSO_4$, *Bst* DNA polymerase (8U) and adding sterilizedddH_2O to a final volume of 25.0 μL. The mixture was incubated at 62℃ for 1 h in water bath and heated at 80℃ to end the reaction for 10 min. The ratio of inner primer to outer primer was 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10, respectively.

Table 1: Primer sequences for LAMP

Primer	Len	Primer sequences (5'→3')
F3	19	TCAGCAAACAACACAAACA
B3	18	TCCGCAATCTTAGCTGTG
FIP	50	CAAACCTTCAATGCCTGTATAGGTAAAAGGTT TTGATATCCTTACTTCAG
BIP	42	ATGTTGCAAATGAGCGTGATAAGAAGCACCTA AACCAAAGCC

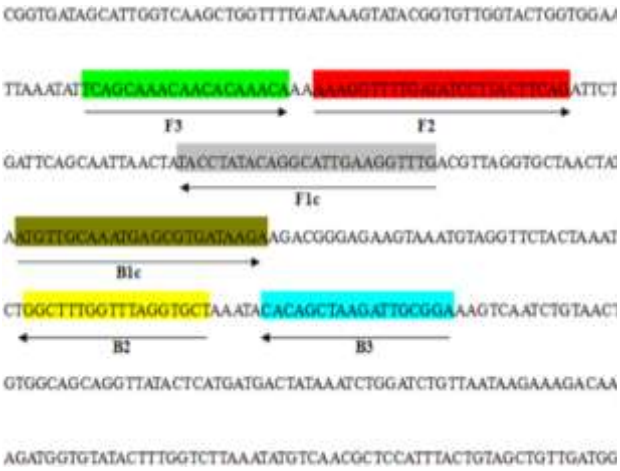

Fig. 1: Sequences of LAMP primers in *OMP P2* gene of *Hps*

The concentration of $MgSO_4$ was 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mmol/L, respectively. The concentration of dNTP was 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mmol/L, respectively. The amount of *Bst* DNA polymerase (8U) was 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.5 μL, respectively. The LAMP was carried out at 55℃, 56℃, 57℃, 58℃, 59℃, 60℃, 61℃, 62℃, 63℃, 64℃ and 65℃, respectively. The LAMP reaction time was 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 min, respectively. In each LAMP reaction, the negative control was set, using sterilized ddH_2O instead of template DNA. After reaction, a total of 5.0 μL LAMP products were electrophoresed on 1% agarose gel.

Specificity Test

According to the optimized LAMP reaction conditions, the DNA of *Actinobacillus pleuropneumoniae*, swine *pseudorabies virus*, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *porcine parvovirus*, *Transmissible gastroenteritis virus*, *Mycoplasma hyopneumoniae* and *Hps* were used as templates and the isothermal amplification was carried out at 60℃ for 45 min and terminated at 80℃ for 10 min. After amplification, a total of 5.0 μL LAMP products were electrophoresed on 1% agarose gel to examine the specificity of LAMP.

Sensitivity Test

The concentration of DNA extracted from *Hps* strains were determined using UV spectrophotometric method and it was 2000 ng/μL. Then the extracted DNA was diluted from 10^0 to 10^{-8} at 10-fold dilution. The templates used for PCR and LAMP amplification were the 10-fold serial diluents of DNA (1:1, 1:10, 1:100.1:10^8). A total of 5.0 μL LAMP products were electrophoresed on 1% agarose gel to examine the specificity of LAMP. The minimal detectable concentrations of PCR and LAMP were determined, respectively.

Results

Determination of Optimal Reaction Conditions

After the LAMP products were analyzed by 1% agarose gel electrophoresis, the optimal LAMP reaction system was determined according to the integrity and clarity of electrophoresis bands. As shown in Fig. 2, Fig. 3, Fig. 4 and Fig. 5, the optimal LAMP reaction system was as follows: the optimal ratio of the inner primer to the outer primer was 1:8; the optimal concentration of magnesium and dNTPs was 3.0 mmol/L; the optimal amount of *Bst* DNA polymerase was 0.8 μL. The specific bands appeared in LAMP products amplified at 60℃ for 45 min. Therefore, the optimal LAMP reaction temperature and time was 60℃ and 45 min, respectively (Fig. 6 and Fig. 7).

Specificity of LAMP

The results showed that the specific ladder bands appeared in amplification products only when *Hps* DNA was used as templates; the DNA of other strains could not be detected. These illustrated that using the LAMP method to detect *Hps* was high specificity (Fig. 8).

Sensitivity of LAMP

As shown as Fig. 9 and Fig. 10, the minimum detectable concentration in the PCR and LAMP reactions were 1×10^{-7} and 1×10^{-5}, respectively indicating that the LAMP had sensitivity levels 100 times higher than that of PCR.

Discussion

The LAMP is a new DNA diagnostic technology and presents a good application prospect in diagnosis of pathogens. The LAMP has high sensitivity for detection of serogroupsO111, O26, and O157 of Vero toxin (VT) - producing *Escherichia coli* (Hara-Kudo *et al*., 2007). Using the LAMP method, the *Salmonella* can be successfully detected in negative egg samples detected by PCR and the bacterial concentration is 2.8 CFU/tube (Hara-Kudo *et al*., 2005). The LAMP method is feasible in early infection for the rapid diagnosis because it can detect *Pseudomonas putida* DNA not only from pure cultures but also from the pathogen in infected fish tissues (Mao *et al*., 2012). According to the sequences of *BCSP31* gene, the specific primers for LAMP have been designed and used for the detection of brucellosis in milk samples (Ohtsuki *et al*., 2008). The sensitivity of LAMP is 1000 times higher than that of conventional PCR when the LAMP is used for detection of *Streptococcus pneumonia* (Mitsuko *et al*., 2005). Based on six distinct regions of the *nuc* gene, specific primers for LAMP have been designed. The detection of *Staphylococcus aureus* can be performed within 45 min through by using LAMP method (Tie *et al*., 2012). In addition, this method was applied to the detection of *porcine circovirus* and H5 subtype *avian influenza virus* (Shen *et al*., 2013; Bao *et al*., 2014).

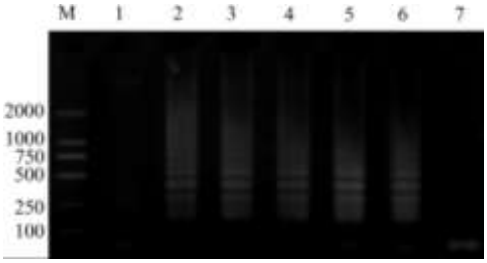

Fig. 2: Optimization of ratio of inner primer to outer primer
M: DL2000 DNA Marker; 1~6: 1:10, 1:8, 1:6, 1:4, 1:2 and 1:1; 7: negative control

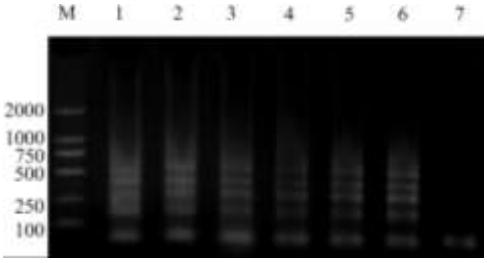

Fig. 3: Optimization of concentration of magnesium
M: DL 2000 DNA Marker; 1~6: 0, 1, 2, 3, 4 and 5 mmol/L; 7: negative control

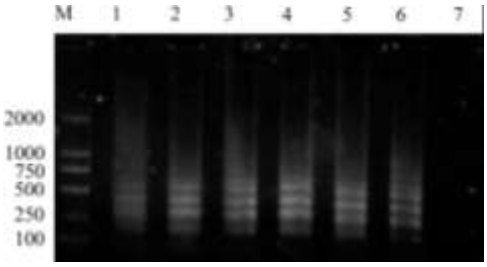

Fig. 4: Optimization of concentration of dNTP
M: DL 2000 DNA Marker; 1~6: 0, 1, 2, 3, 4 and 5 mmol/L; 7: negative control

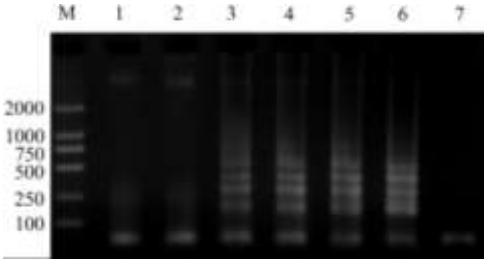

Fig. 5: Optimization of amount of *Bst* DNA polymerase
M: DL 2000 DNA Marker; 1~6: 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 μL; 7: negative control

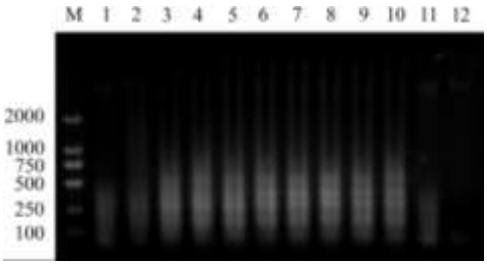

Fig. 6: Optimization of temperature
M: DL 2000 DNA Marker; 1~11: 55℃, 56℃, 57℃, 58℃, 59℃, 60℃, 61℃, 62℃, 63℃, 64℃and 65℃; 12: negative control

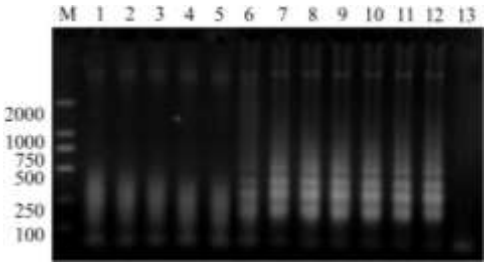

Fig. 7: Optimization of time
M: DL 2000 DNA Marker; 1~12: 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 min; 13: negative control

The LAMP technology for detection of *Hps* was developed in this study. The suitable primer is a key factor effecting the LAMP reaction. Thus, the specific primers for LAMP were designed according to the conserved sequences of *Hps OMP P2* gene. Moreover, the conserved regions of *OMP P2* gene can ensure specificity of LAMP reaction; and the right structure of primers ensures the formation and extension of stem-loop DNA. In addition, the ratio of inner primer to outer primer, concentrations of magnesium, dNTP and *Bst* DNA polymerase as well as the amplification temperature and time are important factors in the reaction system of LAMP.

Comparing with conventional PCR, the advantages of the developed LAMP method are low cost, rapid, easy to operate, highly sensitive and specific. The LAMP reaction can be carried out at 60℃and it does not require an expensive PCR instrument.

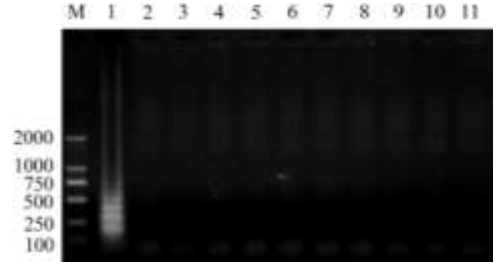

Fig. 8: Specificity of LAMP
M: DL 2000 DNA Marker; 1: Haemophilus parasuis; 2:*Actinobacillus pleuropneumoniae*; 3:swine pseudorabies virus; 4:*Escherichia coli*; 5: *Salmonella*; 6: *Staphylococcus aureus*; 7: *Streptococcus agalactiae;* 8: porcine parvovirus; *9: Transmissible gastroenteritis virus;* 10: *Mycoplasma hyopneumoniae*; 11: negative control

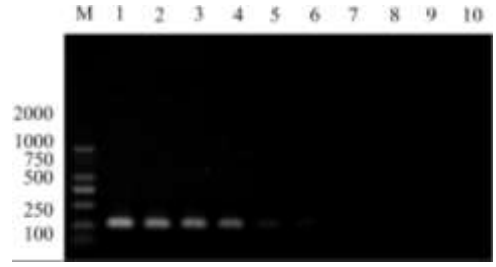

Fig. 9: Sensitivity of PCR
M: DL 2000 DNA Marker; 1~9: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}; 10: negative control

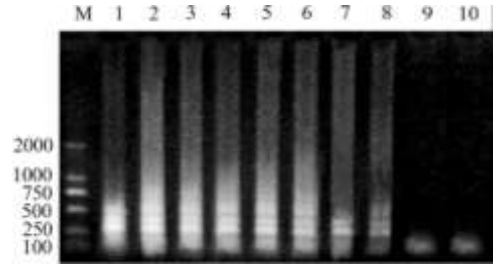

Fig. 10: Sensitivity of LAMP
M: DL 2000 DNA Marker; 1~9: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}; 10: negative control

In addition, the whole reaction can be accomplished within 45 min, while the normal PCR reaction requires at least 2~4 h. The 0.2 pg/μL DNA can be detected by the developed LAMP. Therefore, the LAMP is a highly efficient method for detection of *Hps* and is more suitable for the detections in field and grass-roots sectors as well as can provide reference for clinical diagnosis and treatment of *Haemophilus parasuis* disease.

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